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STRUCTURE-ACTIVITY RELATIONSHIPS OF AGENTS MODIFYING CHOLINERGIC TRANSMISSIONS

ANNUAL SUMMARY REPORT NO. 1

J.P. Long, Ph.D.
J.G. Cannon, Ph.D.

September, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701

Contract No. 17-83-C-3010

The University of Iowa
Iowa City, Iowa 52242

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Hemicholiniums, acetylcholine (ACh); Inhibitors of synthesis of ACh, Structure activity relationship of hemicholiniums.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Structure activity relationship studies of hemicholinium (HC-3) analogs are directed toward better understanding bot the spatial aspect of the receptor and interatomic distances between the 2 cationic heads. Two series of compounds involving 4,4' biphenyl and trans/trans-cyclohexyl derivatives are being investigated and both series are potent inhibitors of acetylcholine synthesis. One compound, 4-methyl piperidine derivative in the biphenyl series, is an inhibitor of ACh synthesis.

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SUMMARY OF ANNUAL REPORT NO. 1

The purpose of this research is the synthesis and biological evaluation of analogs of hemicholinium (HC-3). These are agents which decrease the ability of cholinergic neurones to synthesize acetylcholine. The long-range goal of this research is to develop compounds which can be used to antidote excess acetylcholine within a cholinergic synapse. Some possible approaches are (1) decrease the content of acetylcholine within the cholinergic neurone by interfering with synthesis, (2) desensitizing cholinergic receptors at post-synaptic sites, (3) decreasing the release of acetylcholine from the neurone by stabilizing the membrane or via pre-synaptic receptors which when activated will diminish the amount of acetylcholine released into the synapse.

Thus far 3 agents have been prepared and evaluated for activity. Two of the agents, which are quarternary amines, approximate HC-3 in activity and the tertiary amine derivative is approximately 1/500th as active as HC-3. The latter agent is the first active non-quarternary amine to be reported. The biological assay procedures which have been developed to evaluate these agents are as follows:

1. High performance liquid chromatography with electrochemical detection has been introduced to assay tissue levels of acetylcholine. The lower limits of sensitivity is about 10 pM.

2. Thirty minute incubation of 10 mg of rat caudate nucleus with HC-3-like compounds result is decreased tissue levels of acetylcholine. This is a convenient and definitive assay procedure for hemicholinium-like activity.

3. Rabbit sciatic nerve-gastrocnemius muscle preparation is used for <u>in vivo</u> evaluation of neuromuscluar blocking activity.

4. Rat phrenic nerve-diaphragm muscle preparations are used for <u>in vitro</u> assay for inhibition of neuromuscular transmission.

TABLE OF CONTENTS

I. Statement of the problem: The object of this research is the synthesis and biological evaluation of analogs of hemicholinium which is known to decrease the synthesis of acetylcholine. Compounds in this series are known to antagonize choline uptake into the cholinergic neuron and inhibit nicotinic receptors. This is one theoretical approach to decrease the amount of acetylcholine within a synapse.

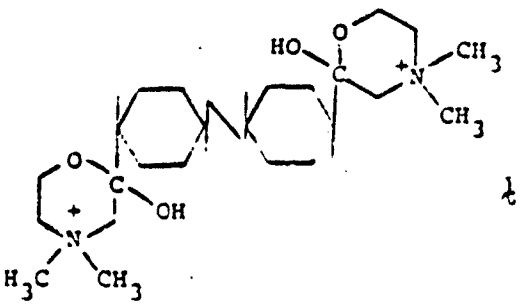
II. Background: At the University of Iowa, Hemicholinium (HC-3) and a large number of analogs were synthesized from 1954-1969. Depending on the structure, a number of the agents were inhibitors of acetylcholinesterase and/or agents which inhibited the synthesis of acetylcholine (HC-3-like).

Selected acetal derivatives of HC-3 antagonized nicotinic agents (nicotine and large doses of acetylcholine) without modifying ganglionic transmission. Also it was noted that selected agents were very toxic (LD-50 for HC-3 in mice is approximately 20 µg/kg). HC-3 is the prototype compound for inhibiting synthesis of acetylcholine and it has been used as a tool in many published studies. Our studies will provide structure-activity relationship data for these very active agents.

III. Approach to the problem: The molecular structures of the agents is designed to provide information concerning interatomic distances between the cationic moieties. Knowledge of optimal substitution on the cationic head will be gained. Biological evaluation will include those synapses known to have considerable turnover of acetylcholine. We will use inhibition of transmission at the neuromuscular junction and inhibition of synthesis of acetylcholine in the caudate nucleus as our major test systems. The toxicity of the compounds will be evaluated, as well as their ability to alter DFP-induced toxicity.

II. Chemistry: Synthesis of Hemicholinium ("HC-3") Analogs

Synthetic work leading to a cyclohexane congener 1 of hemicholinium ("HC-3") has been established on the basis of definition of the geometry of the intermediate 4 (Scheme I).



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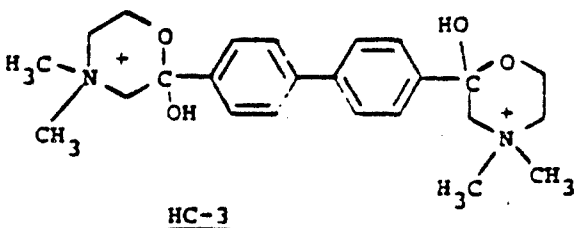

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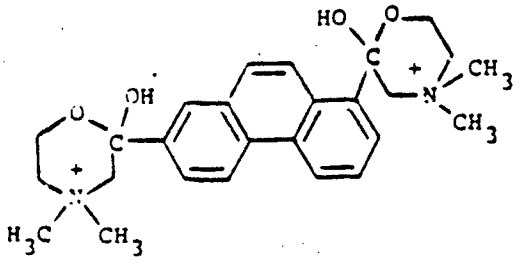
HC-3

Preparation of 1 is shown in Scheme I. The initially formed product 7 of catalytic hydrogenation of dimethyl biphenyl-4,4'-dicarboxylate 6 is a mixture of all possible geometric isomers (traus/trans; cis/trans; cis/cis)[1]. Heating this mixture at elevated temperature in a bomb under reduced pressure induces isomerization to the trans/trans isomer 8[1]. Conversion of the bis-diazoketone 11 into the bis-bromoketone 12 is based upon a method of Wagner and Moore[2]. Schueler[3] proposed that keto aminoalcohols such as 13 (Scheme I) exist in the hemiketal structure as shown for 1 and 2, and indeed, spectral data confirm this structure for 1.

The synthetic approach to the phenanthrene-derived HC-1 congener 14 involves preparation of phenanthrene-1,7-dicarboxylic acid 16, and is shown in Scheme II.



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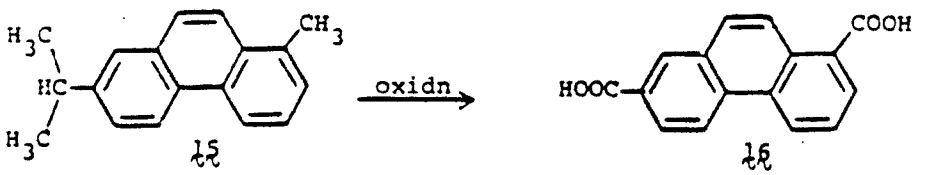
Scheme II. Preparation of Phenanthrene-1,7-Dicarboxylic Acid.

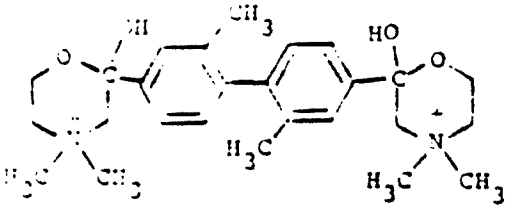


15 16

The starting material retene 15 is available commercially; we have also prepared it by dehydrogenation of abietic acid[4]. Oxidation of retene 15 with $K_3Fe(CN)_6$ by a method of Ruzicka, et al.,[5] gave extremely poor yields of 16, due to an uncontrollable further oxidation of the desired product 16 to a biphenyltetracarboxylic acid. Satisfactory yields of 16 of high purity were attained by oxidation of retene with aqueous sodium dichromate in a bomb at elevated temperature, according to a method of Friedman, et al.[6] This oxidation has now been scaled up and the reaction conditions have been optimized. Ample supplies of 16 will permit elaboration of the carboxyl

... series of MC-3 containers.

In synthetic efforts leading to the 2,2'-dimethylbiphenyl system 17, we have performed the synthetic steps outlined in Scheme III.



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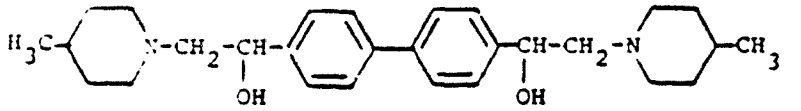
Yields in the step 20 → 21 (Scheme III) are low; we have not yet optimized the reaction conditions. Nevertheless, we obtain preparative quantities of the diolefin 21.

Compounds 22 and 23 (previously prepared in these laboratories[13]) have been resynthesized for further and extended studies. The synthetic route is shown in scheme IV.



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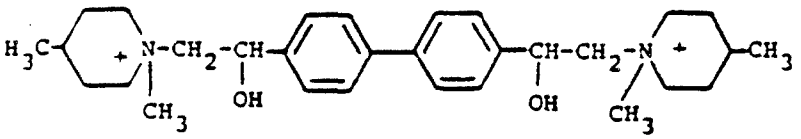

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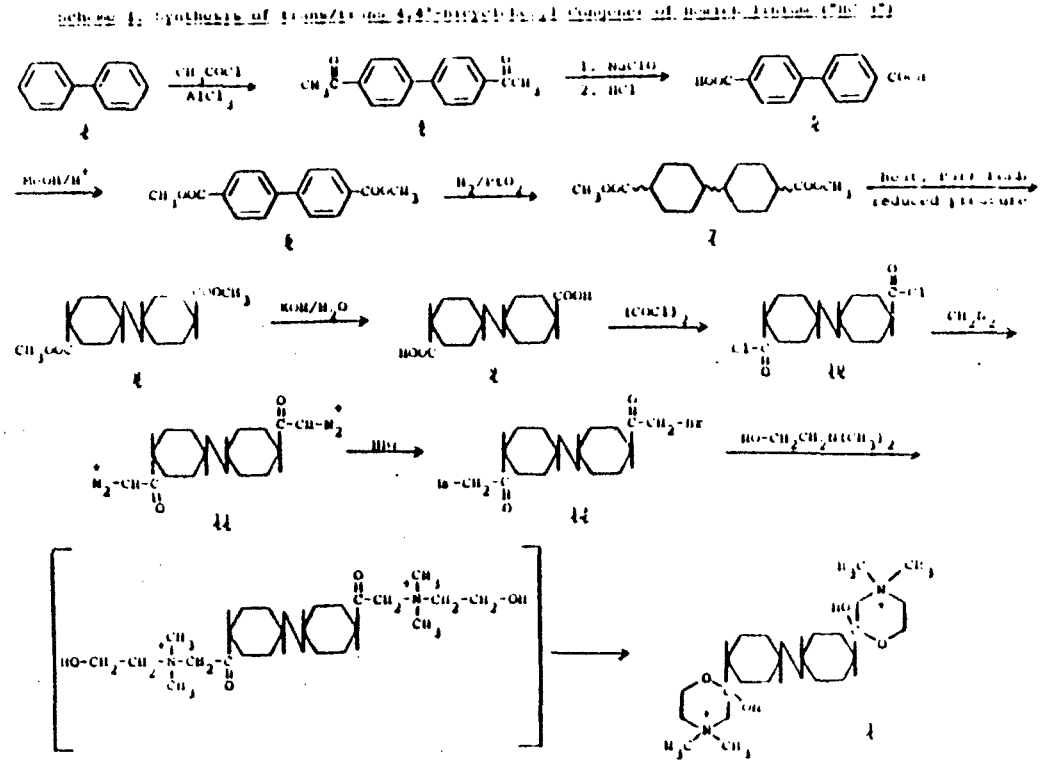
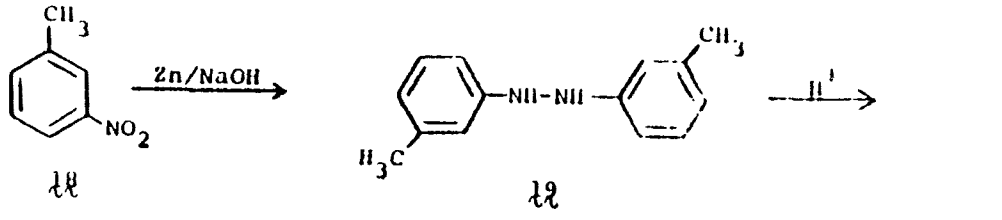
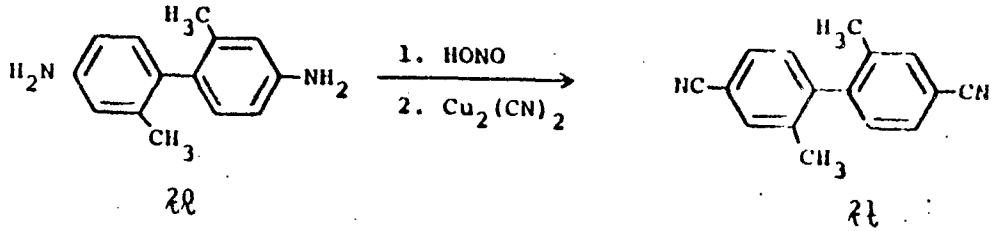


Scheme III. Preparation of 2,2'-Dimethyl-4,4'-dicyanobiphenyl

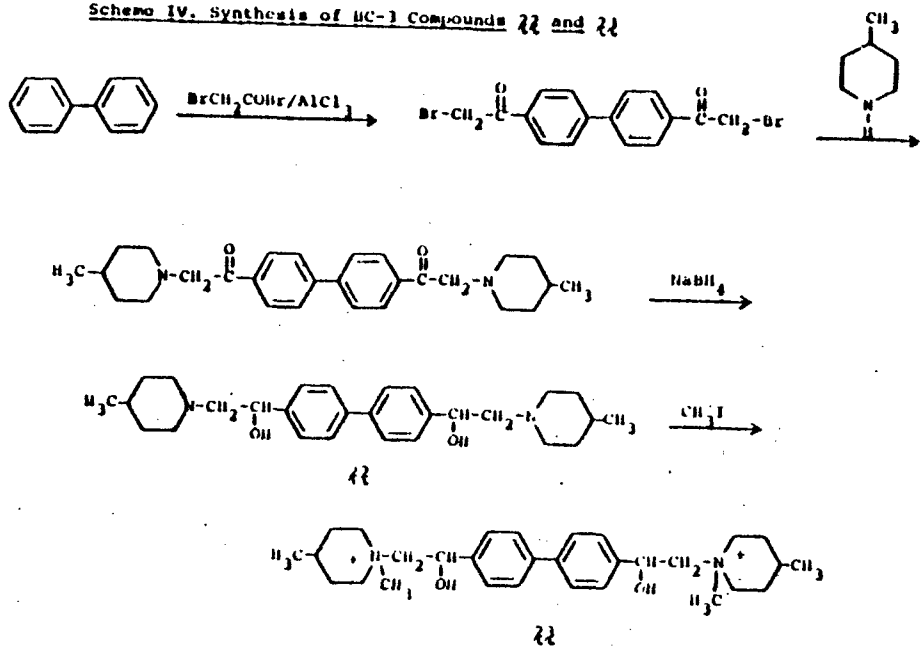


18 19



20 21

Scheme IV. Synthesis of HC-3 Compounds 42 and 22



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22

Experimental

This section describes synthesis of all compounds prepared to date under the Contract.

Melting points were determined in open glass capillaries and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee. IR spectra were recorded on a Beckman IR 4240 instrument. NMR spectra were recorded on a Varian Associates EM-390 instrument using tetramethylsilane as the internal standard. Mass spectra were obtained on a Ribermag 10/10 mass spectrometer. Preparative HPLC was done with a Waters 500 A Prep HPLC apparatus.

4,4'-Diacetylbiphenyl (4). Anhydrous $AlCl_3$ 2.67 g, 0.02 mol) was covered with a mixture of 10 mL of CS_2 and 1.54 g (0.01 mol) of biphenyl. Acetyl chloride (1.57 g, 0.02 mol) was slowly added, and the reaction mixture was gently heated in a water bath until evolution of HCl ceased. The CS_2 was removed under reduced pressure (aspirator) and the residue was treated with ice and excess dilute HCl. The solid which separated was collected on a filter and air-dried. It was recrystallized from EtOH to give 1.97 g (83%) of material, mp 189-190°C. Lit[7] mp 192°C.

Biphenyl-4,4'-dicarboxylic Acid (5). Technical grade calcium hypochlorite (50 g) in 200 mL of H_2O was treated with 35 g of K_2CO_3 and 10 g of KOH in 100 mL of H_2O. The resulting mixture was agitated vigorously until the initially formed gel became fluid. The suspended solid was removed by filtration and was washed on the filter with 100 mL of H_2O which was added to the filtrate. To this solution in a 2L flask was added 3 g (0.0126 mol) of 4, and the mixture was stirred and heated at 75-85°C for 5 h. The reaction mixture was treated with 10 g of $NaHSO_3$ in 40 ml of H_2O, and was then cooled to room temperature. Excess conc HCl was carefully added, and the white solid

which separated was collected on a filter, washed with H_2O, and air-dried. It was recrystallized from EtOH to afford 2.8 g (93%) of a white solid, mp > 250°C. Lit[8] mp > 250°C. IR (KBr) 1759 cm^{-1} (COOH).

Dimethyl Biphenyl-4,4'-dicarboxylate (6). Compound 5 (3 g, 0.0123 mol) in 75 mL of anhydrous MeOH and 2 drops of conc H_2SO_4 was heated under reflux for 14 days. The resulting mixture was cooled, transferred to a separatory funnel, and diluted with 200 mL of H_2O. The resulting mixture was extracted with Et_2O. The ethereal extract was washed with 5% $NaHCO_3$, H_2O, and dried (Na_2SO_4). Evaporation of the Et_2O left a solid which was recrystallized twice from Et_2O to give 2.8 g (85%) of product, mp 205-206°C. Lit[8] mp 214°C. IR ($CHCl_3$) 1729 cm^{-1} (ester C=O). NMR ($CDCl_3$) δ3.98 (s, 6H, OCH_3), 7.26-8.23 (m, 8H Arom H).

Dimethyl Bicyclohexyl-4,4'-dicarboxylate (7). Following a patent procedure[1], 3 g (0.011 mol) of 6 was hydrogenated at 50°C in 100 mL of AcOH in the presence of 0.05 g of PtO_2. When uptake of H_2 ceased, the catalyst was removed by filtration, and the filtrate was diluted with H_2O and neutralized with aqueous Na_2CO_3. A solid separated (3.0 g) which was collected on a filter. TLC analysis (SiO_2, $CHCl_3$) indicated 3 components. IR ($CHCl_3$ 1710 cm^{-1}) (ester C=O). NMR ($CDCl_3$) showed no aromatic H. This material was utilized in the next step without purification.

trans/trans-Dimethyl Bicyclohexyl-4,4'-dicarboxylate (8). A modification of a patent procedure[1] was utilized. Product 7 (2 g, 0.0071 mol) was heated overnight with 0.5 g of NaOH in 12 mL of MeOH and 5 mL of H_2O. The MeOH was removed by distillation, and excess conc HCl was added to the aqueous mixture. The white solid which separated was collected on a filter, washed with H_2O and air-dried. This material was placed in a bomb under reduced pressure (15 mm) and the bomb was heated at 250°C for 2 h, then at 300°C for 2

... material melted at 354°C. Lit[1] mp 355°C. ... suspended in 80 mL of MeOH and 2 mL of conc H_2SO_4 and was heated overnight under reflux. The reaction mixture was poured over excess ice and the solid which separated was collected on a filter, washed with H_2O, air-dried, and recrystallized from 9:1 MeOH-H_2O to afford 1.46 g (73%) of white crystals, mp 112-113°C. Lit mp 116°C[1], 100-101°C[9]. HPLC analysis (SiO_2, toluene-... 1:1) indicated the material to be homogenous. NMR ($CDCl_3$) δ 1.0-2.3 (m, 20H, aliph H), 3.6 (s, 6H, CH_3). IR (KBr) 1690 cm^{-1} (C=O).

trans/trans-Bicyclohexyl-4,4'-dicarbonyl Chloride (10). A solution of 4.0 g (0.014 mol) of 8 and 8 g of NaOH in 100 mL of MeOH and 80 mL of H_2O was heated under reflux for 12 h. The MeOH was removed under reduced pressure, and the aqueous solution was acidified with conc HCl. The solid which separated was collected on a filter, washed with H_2O, and carefully air-dried.

To 0.8 g (0.003 mol) of this material was added over 5 min 25 mL of $SOCl_2$, and the resulting mixture was heated at 150°C for 12 h. Unreacted $SOCl_2$, was removed by repeated azeotroping with benzene, to leave 0.86 g of a brown oil. IR (neat) 1785 cm^{-1} (COCl). This material was used in the subsequent step without further treatment.

trans/trans-4,4'-bis(Diazocarbonyl)bicyclohexyl (11). A solution of 1.5 g (0.005 mol) of crude 10 in 100 mL of anhydrous tetrahydrofuran was added dropwise over 15 min at 0°C to a stirred solution of 2 mL of triethylamine and alcohol-free diazomethane (prepared from 21.5 g of Diazald[10]) in 100 mL of anhydrous Et_2O. Stirring was continued for an additional 3 h under N_2. The reaction mixture was filtered, and the solid on the filter was washed with several portions of anhydrous tetrahydrofuran. The combined filtrate and washings were evaporated under reduced pressure, and the residue was chromatographed on SiO_2 by a dry column technique and was eluted with ethyl

acetate/benzene/$CHCl_3$ (2:4:4). The solid obtained from the eluate was recrystallized from benzene to afford 1.2 g (80%) of fine crystals, mp 159-160°C. IR (KBr) 2100 (N=N), 1720 cm^{-1} (C=O). NMR ($CDCl_3$) δ 0.8-2.0 (m, 20H, aliph H), 5.2 (s, 2H, $COC\underline{H}N_2$. MS m/e 302 (M^+).

Anal. Calcd for $C_{16}H_{22}N_4O_2$ c, 63.58; H, 7.28; N, 18.54. Found: C, 63.63; H, 7.59; N, 18.42.

trans/trans-4,4'bis-(bromoacetyl)bicyclohexyl. (12). To a solution of 0.25 g (0.0006 mol) of 11 in 10 ml of pentane was added with stirring at room temperature 20 mL of 48% aqueous HBr, and the reaction mixture was permitted to stand several hours until evoluation of N_2 ceased. The pentane layer was washed with H_2O to remove HBr. The combined aqueous washes were neutralized with 5% $NaHCO_3$, and this mixture was extracted with $CHCl_3$. The combined $CHCl_3$ extract and pentane solution was evaporated under reduced pressure, and the residue was chromatographed on SiO_2 and eluted with 9:1 benzene/ethyl acetate. Evaporation of the eluate gave a light yellow solid which was recrystallized from toluene to give 0.164 g (67%) of product, mp 148-149°C. NMR ($CDCl_3$) δ 0.8-20.0 (m, 20H, aliphatic H), 4.1 (s, 4H, $COC\underline{H}_2Br$). IR (KBr) 171 cm^{-1} (C=O).

Anal. Calcd for $C_{16}H_{24}Br_2O_2$ c, 47.06; H, 5.88; Br, 39.92. Found: C, 47.36; H, 5.74; Br, 38.80.

trans/trans-4,4'bis-{2-(2-hydroxy4,4-dimethyl-1,4-tetrahydrooxazinyl)} bicyclohexyl (1). To 0.1 g (0.00025 mol)of 12 in 15 mL of anhydrous tetrahydrofuran, under N_2, was added with stirring at room temperature 0.45 g (0.005 mol) of dimethylamincethanol in 2 mL of anhydrous tetrahydrofuran. The reaction mixture was stirred for an additional 1 h. The solid which separated was collected on a filter and was recrystallized from EtOH-Et_2O three times to afford 0.143 g (98%) of white crystals, mp 238-240°C. IR (KBr) 3200

[illegible]. NMR ($CDCl_3$) δ 0.5-2.0 (m, 20H, aliph H), 3.0-3.5 [illegible], CH_2).

Anal. Calcd for $C_{24}H_{46}Br_2N_2O_4$ (Karl Fischer H_2O 6.26%) C, [illegible]; H, [illegible]; N, [illegible]. Found C, 46.50; H, 8.04; N, 4.78

Phenanthrene-1,7-Dicarboxylic Acid (16). 1-Methyl-7-isopropylphenanthrene ("retene") 15[4] (15 g, 0.06 mol) and 108 g (0.36 mol) of $Na_2Cr_2O_7 \cdot 2H_2O$ in 270 mL of H_2O were heated in a bomb at 250°C for 20 h. The cooled contents of the bomb were filtered, and the filtrate was acidified with 18% HCl. A white solid separated which was collected on a filter and washed with H_2O and air-dried to afford 12 g (75%) of crude acid product. This material was characterized as its Me ester. A 1.7 g (0.006 mol) sample was treated with excess CH_2N_2 in Et_2O. Evaporation of volatiles provided a solid residue which was purified by HPLC (SiO_2/CH_2Cl_2) to provide 0.52 g (30%) of a white solid, mp 152-153°C. Lit[4] mp 151-152°C. MS m/e 294 (M^+). NMR ($CDCl_3$) δ 4.03 (d, mH, OCH_3), 7.25-9.0 (m, 8H, arom H).

2,2'-Dimethyl-4,4'-diaminobiphenyl (20). A mixture of 100 g (0.72 mol) of m-nitrotoluene, 168 g (4.2 mol) of NaOH, 140 g (2.14 g atom) of Zn dust, 370 mL of H_2O, and 1 L of MeOH was heated under reflux with stirring for 12 h. The hot reaction mixture was rapidly filtered through a sintered glass filter and the heavy emulsified filtrate was extracted twice with Et_2O. The combined ethereal extracts were washed with H_2O and the organic layer in a 5 L flask was treated with 1.8 L of EtOH and 280 mL of conc HCl. The resulting mixture was heated on a steam bath to remove Et_2O, then it was heated under reflux for 1 h. The reaction mixture was cooled and the solid which separated was collected on a filter. It was washed with EtOH to give 40.3 g (39%) of a white solid. A small amount of this was treated with NH_4OH to liberate the free base which was recrystallized from petroleum ether (bp 35-60°C) to give

colorless crystals, mp 99-100°C. Lit[11] mp 101-102°C. NMR ($CDCl_3$) [illegible] (s, 6H, CH_3), 3.29 (s, 4H, NH_2), 6.39-6.96 (m, 6H, arom H).

4,4'-Dicyano-2,2'-dimethylbiphenyl (21). Compound 20 (14 g, 0.066 mol) was diazotized in 200 mL of H_2O and 41 g of conc H_2SO_4 with 9.1 g of $NaNO_2$ at 5°C. $Cu_2(CN)_2$ was prepared according to a procedure of Theilacker and Ozerowski[12]. To this $Cu_2(CN)_2$ suspension in H_2O was slowly added, with manual stirring, the diazotized solution. Frequent heating on a steam bath diminished frothing. The temperature was brought to 75°C and was maintained with manual stirring for 40 min. The reaction mixture was filtered and the filter cake was washed with 500 mL of 3M H_2SO_4, then with 1 L of H_2O, then it was air-dried. The dried cake was transferred to a Sohxlet thimble and it was extracted with EtOH for 12 h. The EtOH extract was transferred to a large separatory funnel and it was diluted with H_2O to throw out a yellow solid. This mixture was extracted repeatedly with Et_2O. The pooled extracts were extracted with 3 portions of dil NaOH, one portion of 3N HCl, and 3 portions of H_2O. The Et_2O was evaporated to leave a brown syrup which was mixed with 20 mL of EtOH. This mixture was heated to boiling, and upon cooling slightly a brown tar was deposited. The supernatant was decanted, and upon further cooling, a yellow solid separated, mp 106-108°C. Lit[12] mp 113°C. Yield: 3.5 g (23%). NMR ($CDCl_3$) δ 2.03 (s, 6H, CH_3), 7.07-7.56 (m, 6H, arom H).

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V. Methods - Biology

1. HPLC-EC Assay for Acetylcholine

A method for the determination of acetylcholine has been developed using HPLC with electrochemical detection (HPLC-EC) based on the method of Potter et al (1983). This method uses reverse phase HPLC to separate acetylcholine and choline. The effluent emerging from the column is mixed with acetylcholinesterase and choline oxidase. This mixing is post-column. Choline and choline produced by the hydrolysis of ACh is converted by choline oxidase to betaine and hydrogen peroxide. Hydrogen peroxide production is then monitored electrochemically.

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2H_2O_2$$

Protocol

The mobile phase consists of 0.01 M sodium acetate buffered to pH 5 with 0.02 M citric acid containing 5.0 mg/liter sodium octyl sulfate and 1.2 mM TMA. The enzyme solution consists of 0.2M sodium phosphate buffer pH 8.5 to which is added 1 unit/ml choline oxidase and 2 units/ml of acetylcholinesterase. The pumping rates of the two buffers are 0.80 ml/min and 0.05 ml/min respectively to bring the pH in the reaction coil to pH 8 (optimum for acetylcholinesterase).

The assay system consists of Rheodyne 7125 injector with a 100 µl sample loop, and a Supelco C-18 15 cm deactivated for basic compounds reverse-phase column. The effluent from the column flows into a teflon tee connector where it mixes with the enzyme solution and enters a reaction coil of 30 m of 30 gauge teflon tubing. This coil provides a 2.5 min delay to allow for enzymatic reactions. The effluent then passes a platinum electrode: the potential is set to +0.5 V versus a

Ag:AgCl$_2$ reference electrode for the detection of H_2O_2. The oxidation is measured using a BAS LC-4B detector.

ACh and Ch standards are prepared daily in 0.01 perchloric acid (PCA). Ethylhomocholine (EHC) is used as the internal standard and tissue samples are homogenized in 0.01N PCA containing 200 pmoles EHC/100 µl and allowed to stand on ice for 15 min. The samples are centrifuged and 100 µl samples injected onto the column. ACh content of 10 mg of tissue is determined.

Results

At the time of this progress report the HPLC method for determination of ACh using (Potter et al) procedure with modifications is sensitive enough to determine ACh content in discrete areas of the CNS. The limit to our sensitivity is approximately 10 pmoles. This sensitivity may be further improved with the addition of a pre or post column pulse dampner to decrease baseline noise.

Preliminary data have shown this method to be a reliable and accurate procedure for measuring ACh levels. ACh content in the caudate nucleus has been measured at 40 nmoles/g tissue following decapitation. This is in agreement with other laboratory results using various other assay methods and decapitation.

Studies are presently being conducted to study the effects of hemicholinium-3 (2) and various other analogs on ACh content in the rat caudate. This procedure involves decapitation and rapid isolation of 0.2 mm slices. These slices are then incubated for various times in either Kreb's buffer or Kreb's buffer with experimental compound. Preliminary results indicate that on incubating the caudate slices with Kreb's buffer only, ACh content is 80-100 nmoles/g tissue. This is

similar to ACh content levels found for microwave killed animals where no ACh degradation occurs. Incubating with 10^{-5}M 2 shows a marked decrease in ACh content. Though data are in preliminary stages, this method of incubating in Kreb's or Kreb's with drug and then extraction in 0.01 N PCA with EHC is a possible method to investigate the mechanisms by which hemicholinium-3 or hemicholinium-3 like compounds produce their biological effects.

Of equal value to the goals of this research, this technique will allow us to state whether or not an experimental compound is 2-like. Compounds may inhibit neuromuscular transmission by various mechanisms, but inhibition of acetylcholine synthesis which is reversible by choline is strong supporcive evidence for 2-like activity. Decrease in tissue levels of ACh will be needed as we attempt to antidote toxicity following inhibition of acetylcholinesterase.

2. Rabbit Neuromuscular Junction

Dutch rabbits weighing between 1.8 and 2.4 kg are anesthetized by intravenous administration of 250 mg/kg of phenobarbital sodium. The trachea is isolated and respiration is supported by a Harvard respiration pump. One of the jugular veins is cannulated for intravenous administration of compounds.

One of the sciatic nerves is isolated and sectioned centrally and bipolar platinum electrodes are placed on the distal end of the sciatic nerve and attached to a Grass S4C stimulator. The parameters of stimulation are as follows: every 10 seconds tetanic stimulation is delivered for 0.2 of a sec at 200 Hz. The pulse duration is 0.2 msec at maximal voltage usually 20 volts. The knee and ankle are attached to a solid mount and the tendon of Achilles is isolated and sectioned. Ten

[illegible] tension is placed on the tendon and [illegible] with a Beckman R-611 recorder. Stimulations are applied continuously for several hours. In most preparations contractions [illegible] after approximately one-half hour. The compounds are [illegible] Increasing doses varied by 0.3 log intervals until dose response curves are obtained. Time to produce maximal inhibition is allowed [illegible] 30 min. Following the highest dose of the compound choline chloride, 5 mg/kg, is administered intravenously to test its antagonistic action against the neuromuscular blockade.

3. Rat Phrenic Nerve-Diaphragm Preparation

Adult rats are killed by a blow to the head and the chest opened and phrenic nerve and a fan-shaped diaphragm on one side including one rib is isolated and placed in Krebs solution. (gms/L-NaCl, 5.54; KCl, 0.35; $MgSO_4 \cdot 7\ H_2O$, 0.29; $CaCl_2$, 0.28; KH_2PO_4, 0.16; $NaHCO_3$, 2.1, glucose, 2.1).

The phrenic nerve-diaphragm is attached firmly to a holding clamp in an isolated bath preparation and bipolar silver electrodes are threaded around the phrenic nerve. The parameters of stimulation are as follows: every 10 sec a frequency of 200 Hz is delivered for 0.1 sec. The pulse duration is 0.2 msec and maximal voltage, usually 10 V. The compounds are added to the bath and concentration is varied by 0.48 log intervals. The time required for additional doses of the compound will be 30-60 minutes. Choline chloride, 10 mg/l is added to test the antagonistic effects of this agent.

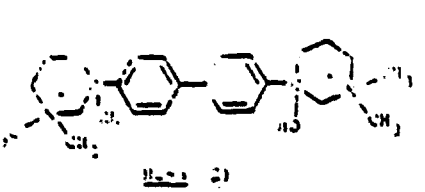
4. Antagonism of Physostigmine Toxicity

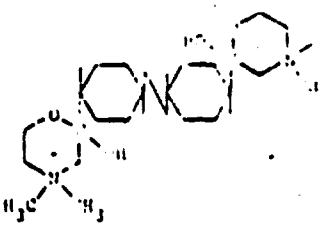
Mice weighing 20-24 g are pretreated 30 minutes with the agent to be tested. Physostigmine is then administered intraperiotoneally in a

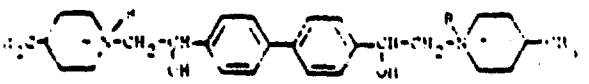
dose of 1.1 mg/kg. This is sufficient to produce 70-80% deaths in nonpremedicated animals. For detailed studies shifts in the entire dose response curve of physostigmine is evaluated.

Results - Active Hemicholinium Analogs.

1. The following compounds are currently being evaluated.







22 R = H
23 R = CH_3

Compound	Inhibition of the rabbit sciatic nerve gastrocnemius muscle. ID50 - µg/kg - IV (95% confidence limits)
HC-3	4.3 (3.4 - 6.1)
22	2,370 (1,880 - 3,050)
23	2.3 (1.5 - 4.4)
1	approx. 6 (n=2)

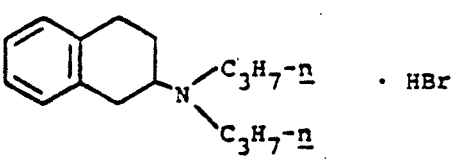
The pattern of action for neuromuscular inhibition appears very similar for all of the above compounds. (See Figs. 1-4) The onset of action is slow and the inhibition of transmission is long (hours). The neuromuscular blockade of all agents is reversed by choline.

We are evaluating the compounds in the rat phrenic nerve-diaphragm preparation. Compound 23 appears to be more active than 2 and 22 is about 1/10 as active. We have no suggestions at this time to explain the apparent differences of relative potency for 22 vs 2 in this preparation vs the rhesus sciatic nerve-gastrocnemius muscle preparation.

2. Antagonism of physostigmine induced toxicity

Apomorphine, 10 mg/kg, administered 0.5 hour before IP administration of physostigmine significantly antagonized the toxicity of physostigmine in mice. Haloperidol, 1 mg/kg, administered with apomorphine prevented the protective action of apomorphine.

The following compound which we have previously reported to be a very active dopamine receptor agonist failed to antagonize the toxicity of physostigmine.



TL-68

Other dopamine receptor agonists will be evaluated. Also all tertiary amines which are 2-like will be evaluated for their ability to modify physostigmine induced toxicity.

[illegible] grant we are continuing synthesis of [illegible] receptor agonists and these compounds will be screened for protective action.

VII. Conclusions

a. The activity of 2 is approximately the same as we found in the past.

b. Compound 22, though only about 1/500 active as 2 in the rabbit nerve muscle preparation, is a tertiary amine. This is the first report that 2-like activity has been observed for a non-quaternary amine. Non-quaternary amines must be developed if they are to be active on the central nervous system following systemic administration.

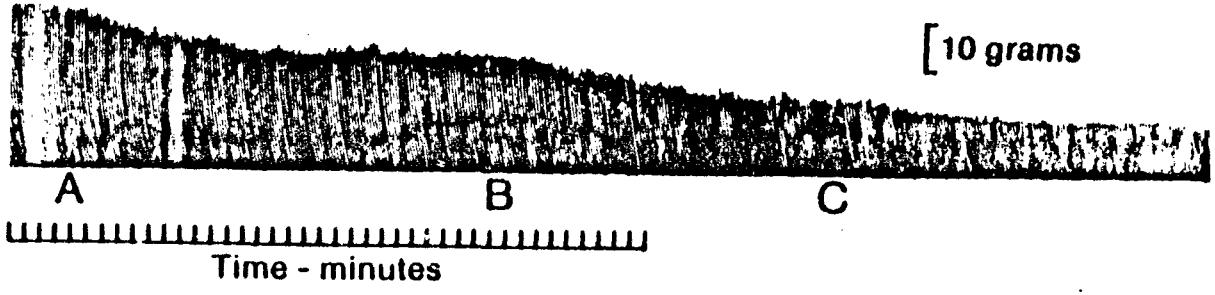
c. Compound 23 appears to be more active than 2. The toxicity of this compound should be marked.

d. Compound 1 is a potent 2-like agent. This chemical structure represents a new structural approach and hopefully we can use it and analogs to better estimate charge and spatial distribution on the receptor. The biphenyl of 2 is not required for activity.

VIII. Recommendations

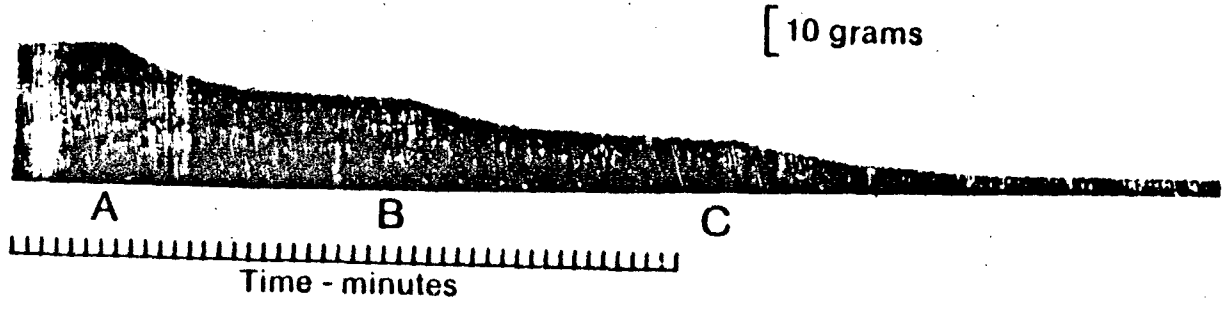
More research data are needed to determine fully the pharmacology of the above chemical entities. Whether the highly active quaternaries 1 or 23 have potential use, we do not know. The discovery of an active tertiary amine, 22, offers the possibility of inhibiting synthesis of acetylcholine within the CNS following systemic administration.

Fig. 1 Inhibition by HC-3 of the rabbit sciatic nerve gastrocnemius muscle preparation. Stimulated once every 10 sec. by 200 Hz for 0.2 sec., pulse duration 0.2 ms., supramaximal voltage



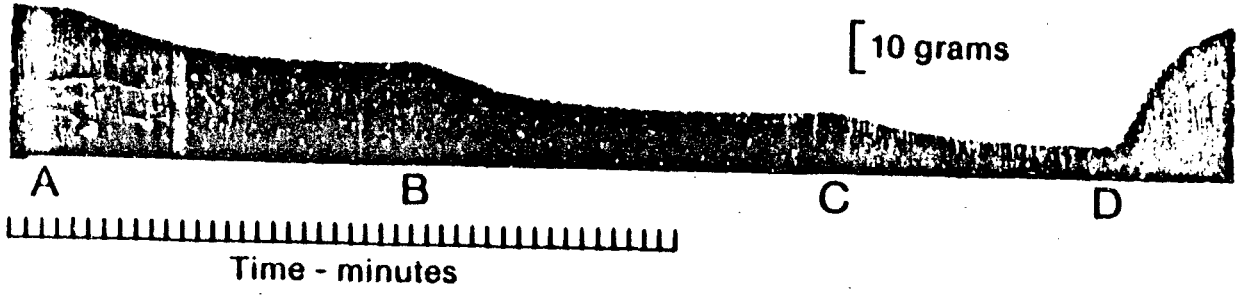
Doses of 2
A - 2 µg/kg
B - 4 µg/kg
C - 8 µg/kg

Fig. 2 Inhibition by A-5 of the rabbit sciatic nerve gastrocnemius muscle preparation. Stimulated every 10 sec. by 200 Hz for 0.2 sec., pulse duration 0.2 ms., supramaximal voltage



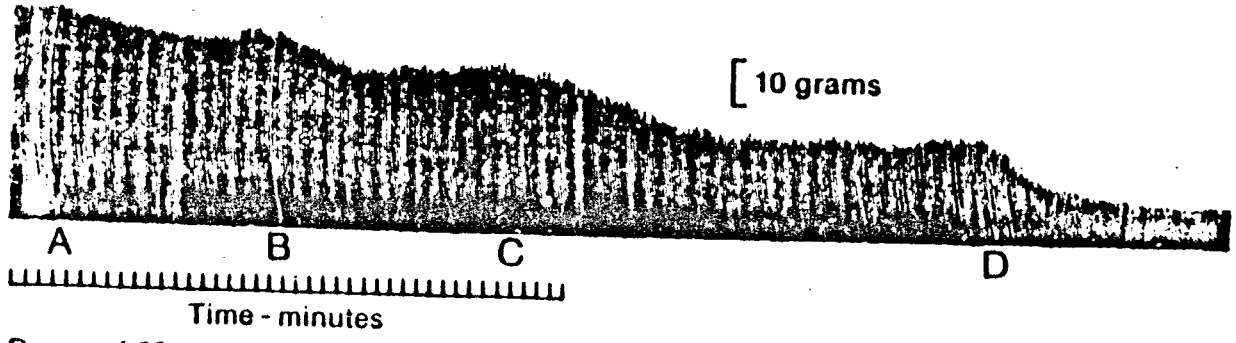
Doses of 23
A - 1.0 µg/kg
B - 2.0 µg/kg
C - 4.0 µg/kg

Fig. 3 Inhibition of rabbit sciatic nerve gastrocnemius muscle preparation by A-5. (Same stimulation parameters)



Doses of 23
A - 1.0 µg/kg
B - 2.0 µg/kg
C - 4.0 µg/kg
D - Choline Chloride - 5 mg/kg

Fig. 4 Inhibition of rabbit sciatic nerve - gastrocnemius muscle preparation by A-4. (Same stimulation parameters)



Doses of 22
A - 1.0 mg/kg
B - 2.0 mg/kg
C - 4.0 mg/kg
D - 8.0 mg/kg